African Journal of Biotechnology Vol. 10(22), pp. 4602-4607, 30 May, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1991
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Somatic embryogenesis and plantlet regeneration from protoplast culture of Muscari neglectum Guss

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Accepted 4 November, 2010

A protocol has been developed for plant regeneration from protoplast culture of Muscari neglectum using regenerable embryogenic calli obtained from bulb culture on Murashige and Skoog medium (MS) plant growth regulators (PGR)-free or containing lower 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) concentrations and 30 g/l sucrose. Protoplasts were isolated directly from embryogenic calli, embedded in Ca-alginate beads and cultured with nurse cells in MS medium supplemented with 1 mg/l each of NAA and BA, 100 mg/l ascorbic acid and 0.5 M mannitol at 25°C in darkness. After 4 weeks of culture, microcalli appeared on the surface of the Ca-alginate beads. Growth of microcalli in the medium with nurse cells (33.3%) was much better than those in the medium without nurse cells (6.5%). Transferring beads onto MS medium supplemented with 0.1 mg/l BA increased the growth of embryogenic calli. Somatic embryo development was observed either on half strength MS medium PGR-free or with 1 mg/l abscisic acid at 25°C under continuous illumination with fluorescent light. Maturated embryos germinated and then converted to plantlets on half strength MS medium containing 1 mg/l BA after 3 months. The plantlets left in the medium produced bulbs after 5 months.

Key words: Ca-alginate beads, Muscari neglectum, nurse culture, plantlet regeneration, protoplast culture, somatic embryogenesis.

INTRODUCTION

The genus Muscari Miller belongs to Liliaceace family, some members of which are cultivated in some countries as garden plants. Muscari neglectum Guss. is one of the ornamental species from the genus and has been used as well as other species of Muscari, for its diuretic and stimulant properties. The root is antiinflammatory, antiallergic, aphrodisiac and pectoral stimulant (Usher, 1974). This bulbous plant has several attractive traits such as dark blue flower colour and vigorous growth, which are desired to be incorporated via somatic hybridization into the other liliaceous ornamental plants (Nakano et al., 2005). The culture and regeneration of protoplasts are important steps in somatic hybridization and realization of genetic manipulation of economically valuable plants. However, for the successful application of these techniques, the availability of efficient procedures for isolation, cultivation and regeneration are prerequisites (Neves et al., 1999). In monocots, culture of protoplasts isolated directly from the plant is difficult (Vasil, 1983; Novak, 1990; Vasil and Vasil, 1992) and protoplasts derived from cell-suspension often, have failed to produce green plants (Maddock, 1987; Lee et al., 1998). Indeed, plant regeneration in these plants has been usually successful when embryogenic calli were used as the source of protoplasts (Vasil, 1988; Isa et al., 1990; Karamian and Ebrahimzadeh, 2001; Nakano et al., 2005). Although, plant regeneration from protoplasts has been reported for several liliaceous ornamental plants such as Asparagus praecox ssp. Orientalis (Nakano et al., 2003), Lilium spp. (Mii et al., 1994; Godo et al., 1996; Nakano et al., 2000; Horita et al., 2002), Hemerocalis hybrida (Fitter and Krikorain, 1981) and Muscari armeniacum (Nakano et al., 2005), no studies on protoplast culture have yet been reported for M. neglectum. The present study reports for the first time the isolation of protoplasts directly from embryogenic calli derived from

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Abbreviations: MS, Murashige and Skoog medium; **BA,** 6-benzylaminopurine; **MES,** 2-N-morpholinoethane sulfonic acid; **NAA,** 1-naphthaleneacetic acid; **PGR,** plant growth regulators.

bulb culture of M. neglectum, culture in Ca-alginate nurse beads and plantlet regeneration.

MATERIALS AND METHODS

Induction of embryogenic callus

M. neglectum bulbs were collected from Hamedan Province, Iran. Bulbs were washed with tap water and surface-sterilized in 5% (v/v) sodium hypochlorite solution for 15 min followed by three rinses with sterile distilled water. Bulb explants were dissected and cultured on MS (Murashige and Skoog, 1962) basal medium PGR-free or supplemented with 0 to 1 mg/l each of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) in different combinations, 30 g/l sucrose and 8 g/l agar. All media were adjusted to pH 5.7 to 5.8 prior to autoclaving at 121°C for 15 min. For embryogenic callus production, all cultures were maintained at 25 ± 2°C in the dark for nearly 6 weeks. The data for embryogenic callus initiation were scored after 6 weeks of culture. Embryogenic callus induction frequency was calculated as the percent of cultured bulbs producing embryogenic callus.

Isolation and culture of protoplasts

Embryogenic calli were incubated in a filter-sterilized enzyme solution consisting of MS medium with 0.1% (w/v) Pectolyase Y-23, 1% Cellulase R-10, 1% Deriselase, 0.1% MES and 0.5 M mannitol at pH 5.7 to 5.8. The mixture was placed on a rotary shaker (90 rpm) for 2 h at 25 ± 2°C in darkness. The incubation mixture was then filtered through a nylon sieve having a 45 µm pore size and washed twice with a washing solution (0.1% MES in 0.5 M mannitol at pH 5.8) to purify the isolated protoplasts. Then, they were layered onto 20% sucrose and centrifuged for 5 min at 100 g. Viable and intact protoplasts floating at the interface were removed with a sterile pasteur pipette and washed twice with protoplast culture medium. The purified protoplasts were mixed gently with 2% sterile Na-alginate solution (Riedel-deHaën) in 0.5 M mannitol at the density of 1 × 105 protoplasts ml-1. These alginate solutions with the protoplasts were added drop by drop with a sterile pasteur pipette into MS medium containing 1% CaCl2 and 0.5 M mannitol. Each droplet immediately formed an alginate bead. The beads were left in this solution for about 30 min to complete gelation and then washed twice with MS medium containing 1 mg/l each of NAA and BA, 100 mg/l ascorbic acid and 0.5 M mannitol. Cultures were incubated in the same medium. For nurse cultures, beads were suspended in the same medium with a high density (1 × 106 cells/ml) of the cells of the same species. Isolated protoplasts were also cultured in the same medium without embedding in Ca-alginate bead as control. All protoplast cultures were incubated at 25 ± 2°C in darkness. Protoplast cultures were subcultured every 7 to 10 days by replacing half of the old medium by an equal volume of fresh medium with lower osmotic pressure. Medium changes were frequently performed by gradual decreasing of concentration of the growth regulators and mannitol. Growth rate was estimated by counting the fractions of cells which divided inside of the beads.

Embryogenic callus formation and plantlet regeneration

After 4 TO 5 weeks of culture, microcalli appeared on the surface of the beads. When these beads were transferred to half strength MS agar medium supplemented with 0.1 mg/l BA, protoplast-derived embryogenic calli larger than 5 mm in diameter appeared. Embryogenic calli were transferred to a half strength MS medium without growth regulators or with 1 mg/l ABA at 25 ± 2°C under continuous illumination with fluorescent light (50 µmol m-2 s-1) for maturation of somatic embryos. Maturated somatic embryos were transferred to half strength MS medium containing 1 mg/l BA at the same condition for germination and plantlet regeneration were achieved in half strength MS medium containing 1 mg/l BA at the same condition. Then the plantlets were transplanted to the greenhouse.

Statistical analysis

Each treatment contained 25 explants and was repeated three times. The data means from all replications were statistically analyzed using the Statistical Analysis System (SAS) program (1999) and separated by Duncan's multiple range test.

RESULTS AND DISCUSSION

Induction of embryogenic callus

After 4 weeks of culture, off-white soft calli were initiated on bulb explants nearly in all treatments. These calli were soft and friable with no morphogenetic potential at first, but after nearly 6 weeks, small granular structures organized from some regions of the calli (Figure 1a). Production of embryogenic callus was initially slow but careful selection of embryogenic regions and frequent subculturing resulted in vigorous proliferation of embryogenic callus which were utilized as source of protoplasts. The highest frequency of embryogenic callus induction (88.2%) was recorded on MS medium PGR-free (Table 1). It seems that increasing NAA and BA concentrations in media negatively affected somatic embryo production and development. In this case, BA showed higher negative effect than NAA.

Isolation and culture of protoplasts

Embryogenic calli were suitable for enzyme digestion and produced the maximum yield of protoplasts, about 5 to 7 × 105 protoplasts g-1 of fresh weight, after 8 - 10 days of subculture (Figure 1b). Isolated protoplasts from embryogenic calli formed a cell wall within 2 to 3 days in Ca- alginate beads (Figure 1c) and were able to undergo cell divisions, which were used to judge the quality of protoplasts. Although control protoplasts without embedding in Ca-alginate gel showed very low cell division, immobilized protoplasts were able to divide better and form cell colonies. The fact that immobilized protoplasts without nurse cells could divide and form a callus suggests that entrapment in Ca-alginate gel is one of the mildest immobilization procedures known, because the gelling agent itself is not toxic and the gelling process is thermo independent. Immobilization improved a protection for fragile cells such as plant protoplasts (Isa et al., 1990; Brodelius and Nilsson, 1980; Karamian and Ebrahimzadeh, 2001; Karamian, 2007). The combination of Ca-alginate bead and nurse culture method improved

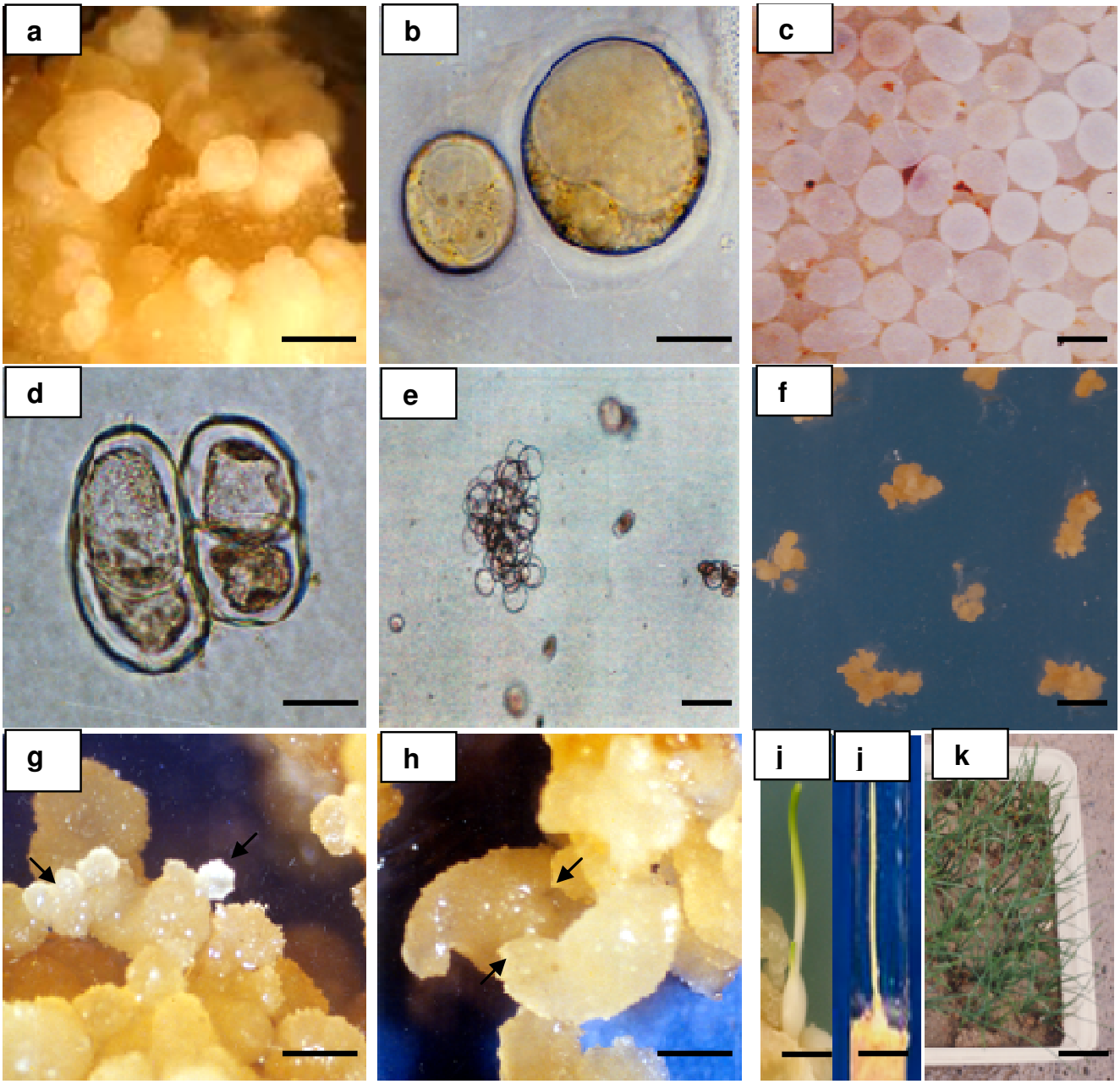

Figure 1. Plant regeneration of protoplast-derived embryogenic calli of M. neglectum. (a) Embryogenic callus induced from bulb explants on MS medium PGR-free containing 30 g·l-1 sucrose after 6 weeks of culture (Bar = 4 mm); (b) isolated protoplasts (Bar = 50 µm); (c) nurses Ca-alginate beads (Bar = 1 cm); (d) first cell divisions in the Ca-alginate beads (Bar = 50 µm); (e) protoplast-derived cell colony (Bar = 100 µm); (f) protoplast-derived embryogeic calli on solid MS medium (Bar = 2 cm); (g) globular and heart-shaped somatic embryos (Bar = 2 mm); (h) bipolar somatic embryos (Bar = 2 mm); (i) plantlet developed from somatic embryo (Bar = 1 cm); (j) regenerated plantlet with bulb (Bar = 1 cm); (k) potted plantlets (Bar = 3 cm).

the protoplast response and reduced the budding phenomenon. The maximum plating efficiency (33.3%) was obtained for protoplasts cultured in Ca-alginate nurse beads (Table 2). It was higher than what was reported previously for M. armeniacum protoplasts embedded in gellan gum and cultured in the same medium (Nakano et al., 2005). The important role of nurse culture was also reported in many species including those of monocots (Isa et al., 1990; Brodelius and Nilsson, 1980; Karamian and Ebrahimzadeh, 2001; Karamian, 2007). In this experiment, the original medium was frequently replaced with a medium with lower osmotic pressure. Protoplast culture is usually successful when medium changes are frequently performed to allow a gradual decrease of the osmotic pressure and concentration of growth regulators. Through the use of Ca-alginate beads, this objective

Table 1. Effect of different concentrations of NAA and BA on embryogenic callus induction of Muscari neglectum after 6 weeks of culture.

Plant growth regulator (mg/l)		Embryogenic callus induction* (%)
NAA	BA	
0	0	88.2 a
0	0.1	62.3 ab
0	0.5	58.2 ab
0	1	42.1 b
0.1	0	40.3 b
0.1	0.1	39.8 b
0.1	0.5	32.7 bc
0.1	1	26.6 cd
0.5	0	25.8 cd
0.5	0.1	23.3 cd
0.5	0.5	16.2 de
0.5	1	14.5 de
1	0	13.8 de
1	0.1	13.1 de
1	0.5	12.3 de
1	1	6.3 e

*Values are the mean for three experiments each of which consisted of 25 replicates. Means in the same column followed by the same letter are not significantly different at $p < 0.05$ according to Duncan's multiple range test.

Table 2. Effect of different concentrations of NAA and BA on the plating efficiency in protoplast culture of Muscari neglectum.

Plant growth regulator (mg/l)		Plating efficiency* (%)		
NAA	BA	Liquid cultured protoplast	Ca-alginate bead	Ca-alginate nurse bead
0.1	0.1	0.0 b	0.0 d	0.0 c
0.1	1	0.0 b	1.3 c	6.8 bc
1	0.1	0.0 b	2.5 b	10.1 b
1	1	1.5 a	6.5 a	33.3 a
10	0.1	0.0 b	2.8 b	16.8 b
10	1	1.3 a	3.1 b	20.0 b

*Values within a column followed by different letter are significantly different at $p < 0.05$ according to Duncan's multiple range test.

could be attained easily and quickly.

Embryogenic callus formation and plantlet regeneration

After 4 to 5 days of subculture, first divisions of embedded protoplasts were observed and cell colonies were produced after 3 weeks (Figures 1d and e). The microcalli appeared after 4 - 5 weeks on the surface of the beads. Transferring of beads to an agar half strength MS medium containing 0.1 mg/l BA increased growth rate of embryogenic calli (Figure 1f). Transferring of embryogenic calli with globular embryos to half strength MS medium PGR-free or with 1 mg/l ABA, resulted in early maturation of embryos. The positive effect of PGR-free medium on somatic embryo development has been reported frequently, especially for the members of Liliaceae and Iridaceae families (Ahuja et al., 1994; Ebrahimzadeh et al., 2000; Suzuki and Nakano, 2001; Nakano et al., 2005). Although its precise action is not clear, ABA was found to induce the expression of maturation genes and to inhibit precocious germination (Williamson et al., 1985; Morris et al., 1990). All the stages of somatic embryogenesis such as globular, heart-shaped and bipolar embryos were observed (Figures 1g and h). Somatic embryo development in the present case was asynchronous and various stages of

Table 3. Effect of different concentrations of BA on conversion of somatic embryos into plantlets in Muscari neglectum after 3 months of culture.

BA (mg/l)	Embryo conversion (%)
0	19.3 c
0.1	20.6 c
0.5	25.0 b
1	32.7 a
2	26.5 b
5	12.7 d

*Values followed by different letter are significantly different at $p < 0.05$ according to Duncan's multiple range test.

globular, heart-shaped and torpedo like embryos could be observed simultaneously in the same embryogenic calli, as previously described for some members of Liliaceae and Iridaceae (Ebrahimzadeh et al., 2000; Karamian and Ebrahimzadeh, 2001; Suzuki and Nakano, 2001, 2003; Karamian, 2007). A few days later, it was possible to isolate bipolar embryos that subsequently germinated on half strength MS medium containing 1 mg/l BA at 25 ± 2°C under continuous illumination with fluorescent light (50 µmol m-2 s-1). Conversion of somatic embryos to plantlets was obtained on the same medium after 3 months of culture (Figure 1i). The plantlets left in the medium produced easily bulbs after 5 months (Figure 1j). Almost all of these plantlets were successfully transplanted to the greenhouse (Figure 1k). The percentage of conversion of somatic embryos into plantlets, according to the BA concentration in the medium, is presented in Table 3. Increasing BA concentration up to 1 mg/l promoted conversion efficiency. With 0 - 1 mg/l BA, the plantlets presented a normal development, but higher concentrations (2 and 5 mg/l) decreased conversion efficiency and promoted abnormalities such as curled and dark green leaves or multiple shooting. The positive effect of lower concentrations of BA on conversion efficiency has been reported in M. Armeniacum (Nakano et al., 2005) and Allium sativum previously (Fereol et al., 2002). However, Nakano et al. (2005) reported that in M. armeniacum, protoplast-derived 3-year-old embryogenic calli did not convert into plantlets and only about 10% of protoplast-derived adventitious shoots produced roots and developed into plantlets after 2 months of transfer to PGR-free medium. It seems that the failure in the conversion of somatic embryos in their study may be partly due to some genetic and/or physiological changes of embryogenic callus cultures used as a protoplast source during long-term subculture. Many aspects can affect the maturation and germination of somatic embryos such as temperature and light conditions (Tremblay and Tremblay, 1991; Firoozabady and DeBoer, 1993), age of explants (Iida et al., 1992) and concentration of growth regulators (Tremblay and Tremblay, 1991). The data reported here demonstrated for the first time the plantlet regeneration from protoplasts derived embryogenic calli of M. neglectum. This effective approach offers the possibility to mass multiply material that has been improved by genetic manipulation experiments.

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